J. Physiol. (1976), **262**, *pp.* 319–348
With 14 *text-figures*
Printed in Great Britain

PROPERTIES OF A FACILITATING CALCIUM CURRENT IN PACE-MAKER NEURONES OF THE SNAIL, *HELIX POMATIA*

BY C. B. HEYER AND H. D. LUX

From the Neurophysiology Laboratory, Max-Planck Institute for Psychiatry, D-8000 Munich 40, Federal Republic of Germany

(*Received 19 February 1976*)

SUMMARY

1. Simultaneous measurements of local voltage clamp currents from patches of soma membrane and K activity at the soma surface were used to analyse the time and voltage dependence of the slow inward current in bursting pace-maker neurones of the snail (*Helix pomatia*).

2. At low levels of depolarization ($\leqslant$ 20 mV) a net inward current is recorded simultaneously with an efflux of K ions from the cell.

3. With larger depolarizations (20–170 mV from holding potential of −50 mV) the deficit in net outward charge transfer compared with K efflux and the appearance of inward-going tail currents following repolarization, reveal a persistent inward-going current also under these conditions. This inward current is carried primarily by Ca ions, as demonstrated by its voltage dependence (a minimum at about +115 mV) and its disappearance in Co-Ringer. It is identified with the slow inward Ca current $I_{\text{in slow}}$ (Eckert & Lux, 1976).

4. The inward current predicted from comparisons of current trajectories reaches a maximum at 15–20 msec (for depolarizations from −50 to 0 mV) and gradually declines with sustained depolarization.

5. Partial inactivation is removed by repolarization to −50 mV and the Ca dependent deficit is greater in the sum of repeated voltage clamp pulses than during sustained depolarization. It is largest for pulses of 25–100 msec duration, decreasing as pulse duration increases.

6. Responses to repeated activation with 100 msec pulses with different repolarization intervals reveal a minimum $I_{\text{in slow}}$ at short intervals (e.g. 20 msec) due to failure to remove partial inactivation. At intermediate intervals (e.g. 200–400 msec) $I_{\text{in slow}}$ shows facilitation. This is revealed in calculations of the net charge transfer and current deficits and is also shown in the tail currents following repolarization. The deficit increases progressively with repetitive stimulation. With longer intervals

(e.g. 800–1000 msec) defacilitation during repeated stimulation after the first two pulses is revealed in calculations of deficits, current trajectories and in the tail currents.

7. Although facilitation depends on duration of repolarization between pulses, increasing intermediate hyperpolarizations from the holding potential of -50 mV are usually ineffective in increasing $I_{\text{in slow}}$. Strong preceding hyperpolarization can even decrease the magnitude of $I_{\text{in slow}}$ and prevent its facilitation with repetitive stimulation, whereas preceding depolarizing pulses can increase $I_{\text{in slow}}$ without preventing its facilitation with repetitive stimulation.

8. The properties of $I_{\text{in slow}}$ are contrasted with previously described membrane conductances and compared with properties attributed to Ca fluxes in other systems.

INTRODUCTION

Several delayed currents have been identified in snail neurones. These include two outward K currents with large depolarizations (Meech & Standen, 1975) and an inward flow of Ca ions ($I_{\text{in slow}}$) at smaller levels of depolarization (Eckert & Lux, 1975, 1976). It has been proposed that the latter also occurs at higher depolarizations where it is obscured by the Kefflux, but can be measured as a deficit in net outward charge transfer under voltage clamp compared with the charge carried by the K efflux (Lux & Eckert, 1974; Lux & Heyer, 1975). Furthermore, Lux & Eckert (1974) have demonstrated that the deficit can increase in the second of two voltage clamp pulses.

In this paper we present the results of experiments using K-sensitive electrodes in conjunction with voltage clamp data to define the deficit and to study its behaviour under a variety of stimulus conditions. The results suggest that the deficit is due to a slow inward current carried by Ca ions and therefore designated $I_{\text{in slow}}$. In addition, the results reveal time and voltage dependence of $I_{\text{in slow}}$ which differs significantly from those previously described for fibre membranes (e.g. Hodgkin & Huxley, 1952*a–d*). The frequency and voltage dependence of facilitation and defacilitation of $I_{\text{in slow}}$ during repetitive activation are described. In the following paper investigations on the relationship between the Ca ions of $I_{\text{in slow}}$ and the control of K permeability in these neurones are presented.

A preliminary account of this work has appeared elsewhere (Heyer & Lux, 1976*a*).

METHODS

Experiments were performed on neurones from the right parietal ganglion of the snail (*Helix pomatia*). Most trials were made on two spontaneously bursting pacemaker neurones regularly found in this ganglion and identified on the basis of physio-

logical properties. Discharge activity recorded with the aid of the patch clamp electrode (see below) reveals patterns typical of these neurones (Fig. 1*A*). Since entirely comparable records can be made before the ganglion is desheathed, it is unlikely that bursting is an artifact of experimental manipulation. One of these neurones, which we designate the 'fast' burster, is obviously identical to a neurone previously described (Kerkut & Meech, 1966; Sakharov & Salanki, 1969). Both intracellular and extracellular records are shown in Fig. 1*A* for this burster. The second 'slow' burster has a longer burst duration and interburst period, measured between the first spikes of successive bursts. Both the maximum frequency of spikes within a burst and the magnitude of the post-burst hyperpolarization were less in slow bursters. The two neurones were further identified

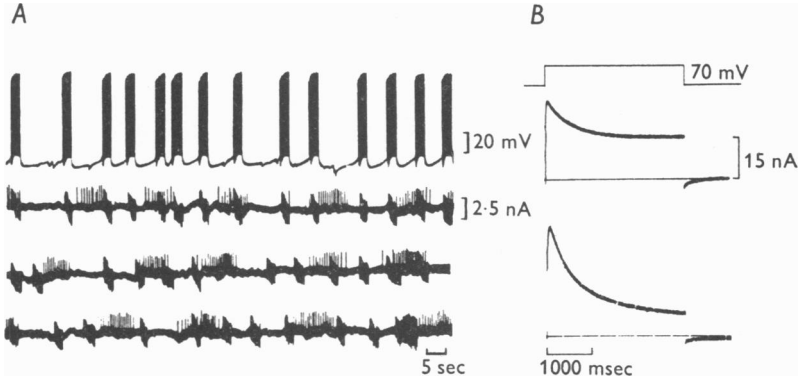

Fig. 1. Characteristics of two bursting pace-maker neurones in the right parietal ganglion of *Helix pomatia*. *A*, spontaneous bursting activity from two neurones in a single preparation. Upper trace: intracellular record from the 'fast burster' showing the short interburst interval, sharp post-burst hyperpolarization and high frequency of firing within the burst characteristic of this cell. Lower traces: continuous record of action currents from the patch pipette situated to record bursts from the fast burster simultaneously with the intracellular record in the upper trace, and the nearby unpenetrated 'slow burster' with its longer bursts and periods between the onsets of bursts and lower maximum rate of firing within the burst. *B*, current trajectories during 3 sec 70 mV voltage clamp depolarizations (from −50 mV; upper trace) showing moderate time dependent depression of the outward current in the fast burster (middle trace) and the more pronounced decline in the slow burster outward current (lower trace).

by their characteristic responses to maintained voltage clamp depolarization (Fig. 1*B*). The depression of the outward current with sustained depolarization was considerably greater in the slow burster. In addition, we found that the deficit current (see below) was much smaller in slow bursters. Most of the data reported in this paper therefore came from fast bursters, with data from the slow burster often serving as a control and especially useful in experiments described in the following paper.

The maintenance of snails before the experiments, the experimental saline solutions and the preparation of neurones for recording were as described by Eckert & Lux (1976), except that the Ca-free Co-Ringer solution contained only 10 mM-Co (mixed immediately preceding the experiment), and pronase was not used to expose neurones

for experiments reported here. Pronase perfused through squid axons interferes with Na inactivation (Rojas & Armstrong, 1971). In six pronase-treated neurones we found responses which indicate pronase may also prolong Ca currents in the somata of snail neurones. Neurones were held at −50 mV between tests, except where noted otherwise, and at least 20–30 sec between stimulus patterns were allowed for recovery of membrane currents (Eckert & Lux, 1976). All signals were stored on magnetic tape for analysis and averaging with a Didac (Intertechnique, Paris) computer when necessary.

Voltage clamp. The voltage clamp circuit, similar to that described by Neher & Lux (1969), consisted of a conventional two-needle clamp (two intracellular microelectrodes) and an additional semi-micropipette on the surface of the soma. Internal electrodes were glass micropipettes, filled with 2 M-KCl and the current electrode (resistance less than 10 MΩ) was shielded to within 150 μm of its tip with a lacquer-insulated coat of conducting silver paint. The reference electrode was an 0·1 M-KCl-Agar bridge in contact with an Ag-AgCl pellet and separated from the bath ground. Clamp currents from a limited area of soma membrane were measured with a 'patch clamp', a semi-micropipette whose interior was held close to ground potential by feed-back control with the internal resistance reduced through adjustment of the feed-back amplification. Thus, during a voltage pulse to the cell interior, the currents which crossed the patch of membrane covered by the pipette (70–120 μm diameter) were measured in the current chamber of the pipette (for details see Neher & Lux, 1969; Eckert & Lux, 1976).

K-sensitive micro-electrodes. Ion sensitive electrodes (2–6 μm outer tip diameter) were fabricated using the basic technique of Lux & Neher (1973). These consisted of a double-barrelled electrode drawn from theta capillary tubing (Duran Glass, Schott, Germany). One side served as a reference barrel and was filled with 100 mM-NaCl solution. The K-sensitive barrel had a column (200–500 μm in height) of ion exchanger (Corning Potassium Exchanger Resin 477317) in the tip. Signals from the two barrels were amplified individually with 'neutralized' capacitance, d.c. amplifiers of high input impedance (10^{13} Ω) and low capacitance. The amplifier for second stage differential amplification, subtracting the reference potential from the recording of the ion sensitive side, was matched to produce a common mode rejection of better than 1:100. In addition to the differential recording single-ended recordings were made against the common silver–silver chloride reference ground electrode to control the potential at the inactive barrel of the electrode. The steady-state response of these double-barrelled electrodes was calibrated in solutions containing known concentrations of KCl (ranging from 3 to 60 mM) in Ringer solution (normally 3 mM-K^+). Calibration curves obtained from these data were used to convert the differential potentials from the electrode into equivalent K^+ concentrations. For some experiments the conversion of the differential recording from the electrode into an equivalent concentration of K^+ was accomplished by feeding the signal into a variable diode function generator (Yokogawa Electric Works, Inc., Tokyo, Japan). The latter was adjusted in ten steps to amplify a ramp voltage signal to values proportional to the calibration curve of the electrodes over a range of 4–60 mM-K^+. This resulted in an output signal from the function generator which was linearly proportional to the calibrated millimolar rise in K^+ above base line (e.g. Fig. 4*C*). Calibration curves for these electrodes in pure KCl solutions had reproducible slopes of about 56 mV for a tenfold change in K^+ concentration. The slope was reduced to between 35 and 40 mV per decade for K^+ ranges of 4–10 mM by interference from other ions (almost solely Na) in the Ringer solution. Response characteristics of these electrodes have been described (Neher & Lux, 1973). These imply that K^+ variations with time courses greater than 10 msec are faithfully reproduced by the sensitive electrode.

We found it necessary to check always the responses of the K^+ electrodes for stronger hyperpolarizing voltages to ascertain that the ratio between outward current and amplitude of the K^+ signal was comparable in both directions of the voltage change. An artifactual voltage depending K^+ signal can appear whereby the electrode response becomes inappropriately large compared with the current during hyperpolarizing voltages. This occurs only when the electrode tip is in contact with or even indenting the visible cell surface. The artifact can be explained by the creation of a K-specific localized and non-rectifying leak conductance (Neher & Lux, 1973). Typical records from recording conditions free of such voltage artifacts are shown in Fig. 2.

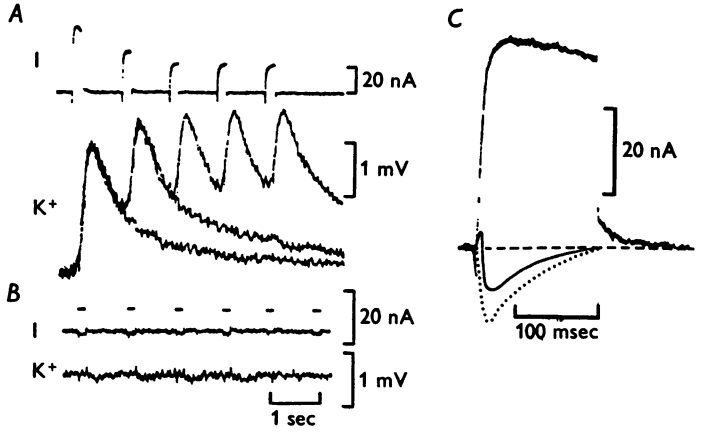

Fig. 2. Simultaneous recordings of voltage clamp currents (I) and K^+ activity at the soma surface (K). *A*, the net outward current (upper trace) declines more rapidly with repetitive depolarizing stimulation than does the K efflux (lower trace) associated with each pulse. Using the K signal for each pulse (examples of the time course of extracellular K following single and double pulse stimulation are shown) the deficit in net charge transfer can be calculated. Compared with the first pulse, values of 12% for the second pulse, and 24–30% for the third and following pulses are obtained. *B*, hyperpolarizing voltage clamp pulses (bars) of same amplitude as in *A* produce small inward currents (upper trace) and appropriately small decreases in K activity but no obvious voltage artifacts in the K record (lower trace, see text for discussion). *C*, the outward pulse 1 current trajectory (presented here with an expanded time scale) together with the current deficits defined by subtracting the pulse 1 trajectory from normalized second and fifth pulse trajectories.

—— $= 1{\cdot}68 \times I_{\text{second pulse}} - I_{\text{first pulse}}$; ----- $= 2{\cdot}28 \times I_{\text{fifth pulse}} - I_{\text{first pulse}}$.

Calculations of the deficit. The total molar amount ($_M$) of K lost from the cell can be compared with the net outward current. To do this the outward current density close to the location of the K^+ measurement was determined for the surface area covered by the current measuring pipette. This value was used to calculate the current for the area of a sphere with a radius of 110–120 μm (which is the approximate radius of these sphere-like neurone cell bodies; see also Kerkut & Meech, 1966). The time integral of this current (I) gives the transferred charge, Q_I, which can

therefore be compared with the charge carried by M, Q_M. Previous comparisons for non-specific cells in which M was derived from sampling with successive measurements the K concentration as a function of time with increasing radial distance gave a ratio, Q_M/Q_I, of $1{\cdot}05 \pm 0{\cdot}35$ (S.D.) (Neher & Lux, 1973).

Although using current densities of the cell soma reduces the problem of local inhomogeneity in current measurement for the entire cell, deviation from radial geometry as well as true differences between net outward and K^+ currents could explain the divergence of this ratio from the value of unity in the individual estimate. From other evidence (Lux & Eckert, 1974) we expected the actual differences between K^+ currents and net currents would be in this range of error. Therefore, a complete determination of M from the distribution of K in the surround of the cell was not carried out. We did use approximative treatments to compare outward charge transfer and detected fluxes of K^+ in a few selected cases (e.g. these approximations were useful for measurements such as those shown in Fig. 3, where the absence of any net outward current precluded calculation of the magnitude of true inward current by the comparative method discussed below). The amount of K^+ions which brings about the recorded change in K concentration and the membrane current can in principle be quantitatively compared at a single known electrode position. We have used two techniques to do this. If the distance d of the K electrode from the cell surface is small compared with the cell radius, the treatment of the problem of diffusion in a semi-infinite space can be applied and

$$dM/adt = D_K dc/dx, r < x < d,$$

in the absence of diffusion barriers (with a the area through which the flux of K takes place, D_K the diffusion coefficient of K and c the increase of its concentration). For small d and sufficient time, dc/dx can be regarded to equal $c_0(\pi D_K t)^{-\frac{1}{2}}$, with c_0 the concentration at $x = r$. Observing that dc/dx changes little with a deviation of no more than 15% for $0 \leqslant x(Dt)^{-\frac{1}{2}} \leqslant 0{\cdot}6$ (Carslaw & Jaeger, 1959, pp. 61 and 72), it can be replaced by $2c/\sqrt{\pi d}$ in this region. A constant current density which produces the flux dM/dt is then approximated by

$$I = FD_K 8\sqrt{\pi r^2 C_T/d}, \quad (1)$$

with C_T the concentration of K at time T. This relation can be used to determine the average K current during the voltage pulse. For the use of (1) the voltage pulse must be of sufficient duration since a time lag of 20–100 msec is usually present in the K readings (Fig. 2), even at very proximal locations of the electrode. The time lag can be attributed to a surface barrier with slowed diffusion. For estimating the surface conductivity (P) of this barrier, the treatment of the diffusion problem with initially constant concentration (Carslaw & Jaeger, 1959, pp. 70–3) was employed and the solution compared with experimental slopes of c. This gave estimates for P of about 1×10^{-3} to 8×10^{-3} cm/sec. If the surface concentration (c_s) of K is responsible for the change in equilibrium potential of the outward current (Neher & Lux, 1973), this change can also be used to estimate P.

The time constant of the change in equilibrium potential is approximated for small t by D/P^2. Such time constants (see also Neher & Lux, 1973) appear to be in the range of tens of milliseconds which would result in estimates of P similar to those calculated above. It was found sufficient for the present estimates to use pulses of 0·3–1 sec and to observe a delay of 100 msec. We used these estimates with very small distances between the electrode and the cell surface only after completion of other studies because of the danger that small dislocations could result in voltage artifacts previously discussed.

To obtain a second estimate of the magnitude of the K currents, c was regarded as

homogeneous in the region $r < x \leqslant r+d$ from which diffusion proceeds into $r+d < x$. Such a condition may hold particularly well if the K signal has reached a steady state and if the electrode is very close to the cell surface, which is covered by a glia sheet of an extension of a few μm. It follows that

$$\int_0^T I(t)\mathrm{d}t = (F4\pi r^2 dc). \tag{2}$$

It is obvious that d is used in different ways in eqns. 1 and 2. Since d is the most difficult parameter to determine, this comparison of values from the two equations may be helpful in determining the approximate magnitude of the K current.

The above calculations can prove useful, especially when there is no net outward current. When a net outward current was present, we used a comparative method to calculate the deficit in net charge transfer. That is, we compared the time integral of the net outward current under voltage clamp (Q) with the K^+ efflux measured as the elevation of the K signal $\int K\mathrm{d}t$ (K_M) above the 4·0 mM background of the Ringer. This time integral of the K signal was taken for a period of 20–100 sec, depending on the speed of the recording and the magnitude of the response; i.e. until the signal could not be differentiated from background levels at a single location for the K-sensitive electrode. If the net outward current represented only the efflux of K^+, then the ratio Q_A/Q_B should be equal to the ratio K_A/K_B, where A and B represent pulses or series of pulses. From Fig. 2 the typical discrepancy between the ratios is obvious. Calculations of the $Q_1/Q_n - K_1/K_n$ for the five pulses give an average deficit of 26 %. This deficit in the net charge transfer of subsequent pulses compared with that of the first indicates the presence of an inward current component. However, an increase of this current component with subsequent pulses cannot be concluded without further inspection of data. An inward current, constant for all pulses 1 to n will appear as an increasing deficit if the outward current decreases. The assumption that a charge displacement H by a constant current accounts for the calculated deficit can be checked from our data. The ratio of the charges carried by the outward K current (K_A/K_B) would equal $(Q_A+H)/(Q_B+H)$. Therefore

$$H = (K_A Q_B/K_B - Q_A)/(1-K_A/K_B).$$

Often calculations of H from our data result in extremely large values. For example, if pulses 1 and 2 for the cell of Fig. 2 are compared, a constant inward current which carries the charge H would need to be 5·2 times the outward current in pulse 1 to account for the apparent deficit in pulse 2. Such a value requires a K current which is several times the measured charge transfer under voltage clamp. An underestimate of the absolute value K_A of this magnitude can be excluded (Neher & Lux, 1973).

In all experiments calculations of H for different pulse numbers or stimulus paradigms with the same neurone gave different values. Estimates of H varied between 0·02 and 15 times the outward current, Q_A. For each cell we re-evaluated the data assuming that the minimum calculated H value for that neurone accurately represented a constant inward current present in all pulses. Although the absolute value of the current deficits were somewhat reduced by using this assumption (often by only 2–3 %), the shapes of the curves in deficit *vs.* voltage and deficit *vs.* frequency plots were essentially unchanged by this technique. Since the true values of the deficit cannot be calculated for Q_A using the comparative method, we have assumed it to be zero for the purposes of presenting figures here.

Because we frequently worked with rather small signals from the ion sensitive electrode (e.g. for depolarizations to near 0 mV), we routinely repeated trials 3–10 times within an experiment. Depolarizations to higher voltages gave larger signals and were accordingly repeated less often. Within an experiment trials of different voltages or stimulus patterns were presented in a random fashion.

In addition to determining this deficit in net charge transfer, a current deficit trajectory was estimated by comparing the shapes of the outward currents in different pulses. To do this the amplitudes of the net outward current at the end of 100 msec in the second and subsequent pulses were normalized and the pulse 1 current subtracted resulting in a predicted net inward current trajectory. Current trajectories can be normalized in two ways for this procedure before subtraction, with the resulting current deficits being slightly different in magnitude. Normalizing the heights of the current trajectories to the same value at 100 msec implies that the Ca current is fully inactivated at that time. Inward tail currents when the neurone is returned to -50 mV at the end of the depolarizing pulse (e.g. see Figs. 8, 13 and 14) indicate that this is not true, and the current deficit calculated in this way slightly underestimates the deficit calculated on the basis of the K^+ data, at least for intermediate and long interstimulus intervals. Alternatively, pulse 1 can be reduced in proportion to the K_1/K_n ratio and then subtracted from pulse n. This assumes that the inward current is of constant magnitude throughout the pulse and results in a trajectory which overestimates the deficit in net transfer. Thus, the real current 'deficit' may be assumed to be between the trajectories calculated by these two methods. Both methods give the same approximate time course for $I_{\text{in slow}}$. Since the first method does not require K electrode data we have used it more frequently here, where we can check accuracy with the K data, to establish its validity as a predictor for use in experiments where K sensitive electrodes are not employed. However, it must be remembered that the apparent return of calculated $I_{\text{in slow}}$ trajectories to zero at the end of 100 msec represents an artifact of this method of calculation. An example of such a calculation is shown in Fig. 2*C*. Pulse 1 has been subtracted from normalized pulse 2 and pulse 3 currents. In this example the deficit calculated for pulse 2 from the difference $(Q_1/Q_2 - K_1/K_2)$ was less than that calculated for pulse 3. The current deficit plots show similar results.

RESULTS

Direct demonstration of a deficit in net charge transfer during small depolarizing steps

There is a comparably small voltage region where the delayed K current is low enough so that the inward Ca current predominates under voltage clamp and the existence of a deficit in net charge transfer is qualitatively obvious. Experiments to record such a condition are technically difficult, however. The removal of glial cells from the surface of the soma, necessary for resolving the very small changes in K activity, often produces a slight increase in leak conductance, obscuring an inward current already partially short-circuited by the delayed K current.

In two particularly favourable preparations we were able to demonstrate the existence of a K efflux in the presence of a net inward current. The results of one such experiment are presented in Fig. 3. Repetitive depolarizations of 18 mV from the -50 mV holding potential resulted in an increase in extracellular K activity. The computer averaged records from the patch clamp recordings indicate a concomitant net inward current. This current appears to increase to a maximum at 150 msec and then declines during the 500 msec pulses.

Estimates of the actual magnitudes of the two currents can be made. To get a lower estimate of the current carried by K^+ ions, the distance of the K electrode from the cell surface and the average increase in K activity at this distance were determined. The use of eqn. (1) (Methods) for this calculation gave an average current of 2·3 nA during the single, 500 msec pulse in Fig. 2. Under the assumption that $d[K]/dx$ was constant for sufficient times (250 msec) after the onset of the pulses, an upper estimate of 2·5 nA was obtained (following eqn. (2), Methods). Both estimates are subject to considerable error. In addition the falling phase of the K signal suggests that K absorption may be present in addition to a diffusion process (see also Lux & Heyer, 1976); this would also result in an underestimate of the K current. Assuming that K efflux is constant with time throughout the pulse, the maximum inward current of approximately 5·5 nA is predicted from Fig. 3 for a small depolarization.

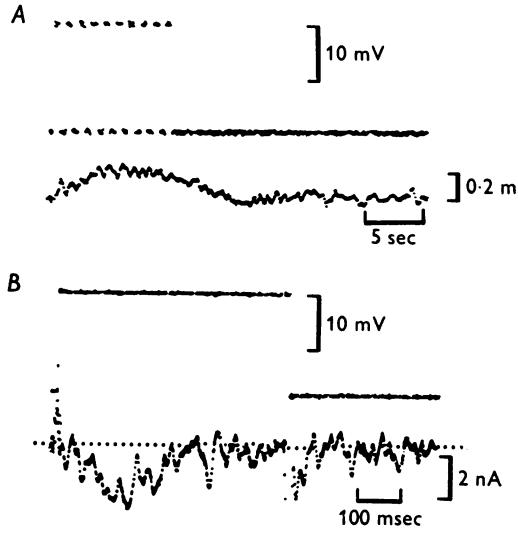

Fig. 3. Demonstration of a net inward current in the presence of a simultaneously recorded potassium efflux. *A*, upper trace: repeated voltage clamp pulses from a holding potential of −50 mV. Lower trace: K^+ activity (computer average of six trains of ten pulses each) recorded at the soma surface. *B*, expanded time scale with the computer average of 60 pulses showing the voltage clamp pulse (upper trace) and the resultant net inward current (lower trace) recorded simultaneously with the K^+ activity in *A*.

Although quantitative comparison is cumbersome because of the small signals involved, it is clear from Fig. 3 that the net charge transfer measured from voltage clamp data significantly underestimates the charge carried by potassium efflux and that the 'deficit current' is not only expressed as a quantitative difference between, for example, the responses to two depolarizing pulses, but is also qualitatively obvious.

Ca dependence of the deficit

The voltage dependence of the deficit and behaviour of Co-treated neurones suggest that the deficit is due primarily to an inward flow of Ca ions.

Voltage dependence of the deficit

A deficit current due to the influx of Ca ions should be zero at the Ca equilibrium potential and a relative minimum deficit can be established by the comparative method. We therefore investigated the voltage dependence of both the net outward current and the K efflux.

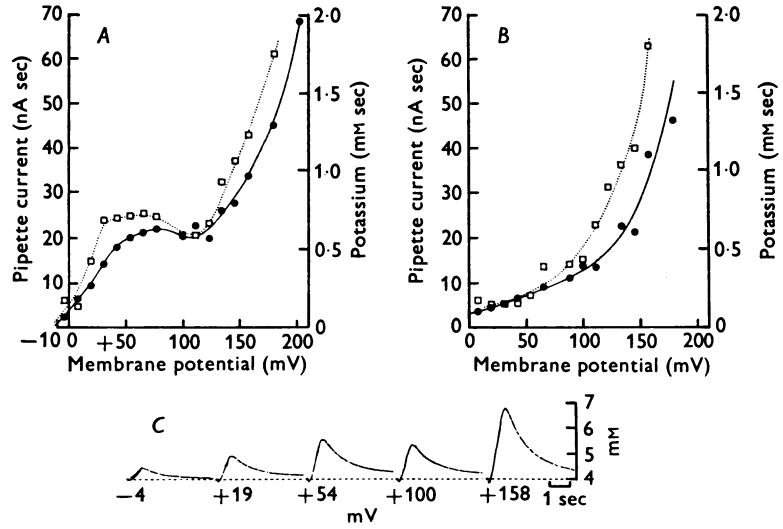

Fig. 4. *A* and *B*, the average net outward current (nA sec; left scale) measured by the patch clamp electrode from the soma membrane during a 500 msec voltage clamp pulse (—●—) and the average elevation in K activity (above the 4 mM of the Ringer; mM sec; right scale) recorded at a nearby K sensitive electrode during the 30 sec interval following the onset of the voltage clamp pulse (---□---) as functions of the membrane potential during the pulse (from − 50 mV holding potential) before (*A*) and after (*B*) treatment with Co-Ringer. Note the N-shape of both K-voltage and net outward current–voltage relationships in *A* and the absence of an N-shape in both plots in *B*. *C*, records of K^+ activity near the soma surface during and following 300 msec depolarizing voltage clamp pulses (from − 50 mV holding potential). Absolute membrane potential during the pulse is indicated at the base of each record. Original K signals have been amplified by the variable diode function generator calibrated to produce a voltage readout linearly proportional to the equivalent K concentration (see Methods). The N-shape in the K-voltage relationship revealed by plots such as the one in Fig. 4*A* is also obvious in the peaks of the signals.

In the region of high positive potentials these *Helix* neurones typically show an N-shaped slope of the *I–V* relationship (Meech & Standen, 1975), such as the one in the diagram of Fig. 4*A*. There is a local minimum between +100 and +125 mV. Typical records of K efflux (linearized K electrode data, see Methods) at different levels of depolarization presented

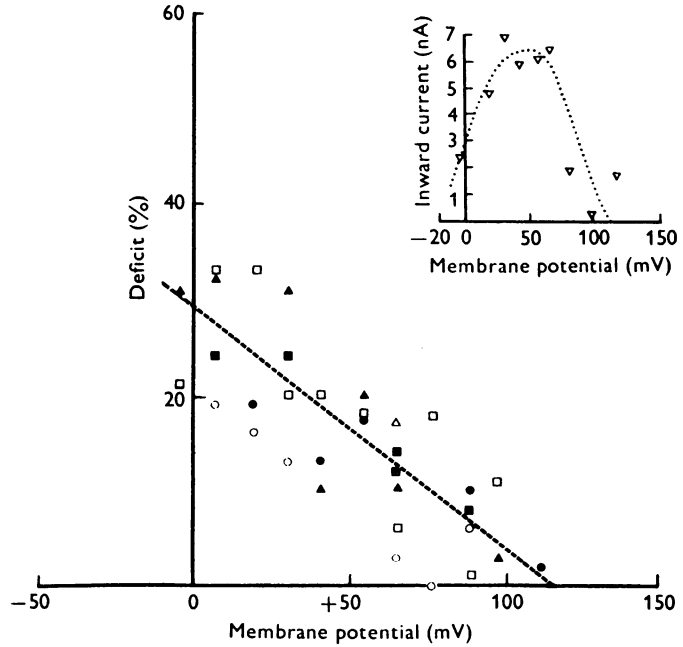

Fig. 5. Voltage dependence of the deficit in net charge transfer calculated from voltage clamp currents and K electrode recordings in five different neurones (all with conspicuously N-shaped *I–V* plots; each neurone represented by a different symbol). For depolarizations of 46 mV or above (membrane potential = −4 mV), the linear regression line drawn by the least-squares fit method for the composite of five experiments crosses the abscissa at a membrane potential of +114 mV. The individual slopes of the deficit voltage plots in the five experiments appear to approach zero between +90 and +135 mV. Inset shows inward current of one neurone (average over 500 msec) as a function of membrane potential for depolarizations from −50 mV. (See text for further discussion of assumptions made in calculating the deficit.)

in Fig. 4*C* reveal the non-linear relationship also between depolarization and K efflux. The relationship is qualitatively similar to that between net outward current and voltage. The *K–V* curve also shows a local minimum between +100 and +125 mV (see Fig. 4*A*). However, there are quantitative differences. The apparent outward current does not increase at the

same rate with increasing depolarizations (up to +100 mV) as does the K signal. This can be expressed as a change in the deficit current (see Methods). If the net outward current represents only the efflux of K^+ ions, then the ratio Q_1/Q_2 (where Q = time integral of I) should be equal to the ratio K_1/K_2 (where K = K efflux). Such is clearly not the case for the data in Fig. 4*A*. The relative deficits were calculated as $Q_1/Q_n - K_1/K_n$, where Q_1 and K_1 were the current and potassium measurements with the highest Q/K ratio, therefore representing the point of minimum deficit. A decrease in deficit with increasing depolarizations is plotted in Fig. 5 from the results of five experiments. The deficit has a minimum at about +115 mV, which is also the local minimum in the I–V and K–V graphs. From such a graph it is clear that at low levels of depolarization the deficit in net charge transfer is considerable and that the net outward current can substantially underestimate the actual charge transfer due to K efflux. In the inset to Fig. 5 the amplitude of the inward current (averaged over 500 msec for a neurone with a large inward current) is represented as a function of membrane potential, using the assumption that the deficit in Q_1 is zero. Because of variations in measured voltage clamp currents among the neurones studied, such calculations of the average amplitude of the inward current are not useful in comparing the results among cells. Therefore, we compare the percentage deficits among neurones.

At voltages above the deficit minimum the K signal again increases more rapidly than the net outward current. We found no artifact of our recording procedure which could account for an apparent overestimate in the K signal, and therefore conclude that the K efflux does increase more rapidly than the net outward current. A similar response is seen throughout the range of depolarizations for Co treated neurones (e.g. see Fig. 4*B*) and is considered in the Discussion.

Effect of Co on the deficit

The minimum deficit at +115 mV suggests that the deficit is due primarily to the flow of Ca^{2+} ions. The effects of substituting Co for Ca in the Ringer support this hypothesis. Fig. 4*B* contains data from the same neurone as Fig. 4*A*, but after the Ca^{2+} in the Ringer was replaced by 10 mM-Co^{2+}. The outward current is clearly depressed from normal for depolarizations below about +160 mV. Above this level the responses are quite similar. Analogously the K efflux is also depressed by Co^{2+} ions. Further, there is no decreasing deficit with increased depolarization.

The deficit current with repetitive activation

The deficit current was originally demonstrated in experiments with two voltage clamp pulses of equal magnitude and duration at fixed intervals

(Lux & Eckert, 1974). We have further investigated the effects of repetitive depolarizing voltage clamp pulses to define the time and voltage dependences of $I_{in\ slow}$ in subsequent pulses.

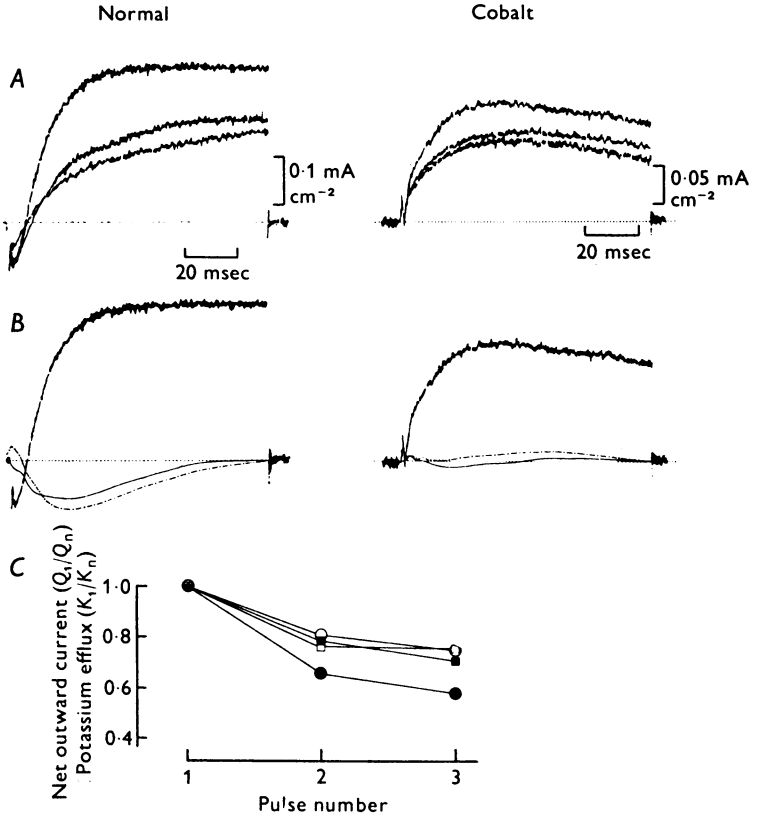

Fig. 6. Superimposed net outward currents (*A*) and calculated current deficit trajectories compared with pulse 1 (*B*) for three 100 msec pulses (from −50 to +7 mV with 900 msec repolarization intervals) from a neurone before (column 1, 'Normal') and after (column 2, 'Co') treatment with Co-Ringer. Current deficits in *B* represent the subtraction of pulse 1 from pulse 2 (——) and pulse 3 (—·—·) trajectories after pulses 2 and 3 were normalized to the amplitude of pulse 1 at 100 msec. (See Methods for further discussion of this procedure.) *C*, normalized values for the net outward current (Q_1/Q_n, circles) and K efflux (K_1/K_n, squares) for the first, second and third pulses in the series before (open symbols, ○, □) and after (filled symbols, ●, ■) Co treatment. The deficit in net charge transfer apparent as the discrepancy between the relative net outward currents under voltage clamp and the net charge transfer carried by K efflux in the normal neurone is abolished by Co treatment.

The current deficit trajectory and the deficit in net charge transfer

In Fig. 6 the responses of a fast burster to three voltage clamp pulses are presented. The net outward current decreases progressively with successive pulses (Fig. 6*A*, column 1). When the heights of the current pulses at the end of each 100 msec depolarization are normalized to the same value and the trajectory of pulse 1 is subtracted from pulses 2 and 3, 'deficits' in the two later pulses are revealed (Fig. 6*B*, column 1; see also Methods).

The decreased rate of rise in subsequent pulses and the corresponding current deficit trajectories calculated result primarily from increased activation of the slow inward current. Simultaneous records of K activity at the soma surface during depolarizing clamp pulses reveal that the decrease in K efflux during the second and third pulses is not as great as the depression of the net outward current with these pulses (Fig. 6*C*). Thus, there is a deficit in net charge transfer ($Q_1/Q_n - K_1/K_n$). It is 23 % of Q_1/Q_2 for pulse 2 and 29% of Q_1/Q_3 for pulse 3 for the neurone in Fig. 6. Similar values were obtained for other trials in this experiment and in other experiments (range 12–34%).

Two additional lines of evidence suggest that the slowed rate of rise in the second and subsequent pulses is due to an increased inward current rather than a change in the kinetics of the outward current.

Comparisons of net outward current records from normal and Co treated neurones during repetitive stimulation suggest that the decreased rate of rise in normal Ringer is due to an increased inward current in subsequent pulses. The effects of Co treatment are obvious from the example shown in Fig. 6: with repetitive stimulation the rate of rise of subsequent pulses does not decrease. Furthermore, for the last pulse in Fig. 6 the net outward current amplitude during the initial 15 msec is increased in Co-Ringer over the amplitude in the cell in normal Ringer. We frequently found that the amplitude of the outward current at the onset of the pulse in subsequent pulses during Co treatment was greater than the outward current amplitude at comparable times in normal neurones. This would be expected from the short-circuiting effect of a slow inward current in normal Ringer such as the one described here with a peak at 15–20 msec after the pulse onset. Elimination of this fast component of the outward current in normal Ringer would not result from a change in the kinetics of the K current unless, for example, an influx of Ca was capable of immediately depressing the Ca independent component of the K current (Heyer & Lux, 1976*b*); i.e. during the initial 15 msec of the pulse. The latter is unlikely.

K electrode measurements can also support the hypothesis that the kinetics of K efflux during the initial part of the pulse are not altered by repetitive depolarizations. On some occasions we were able to move the K electrode very close to the membrane so that the response time of the K signal was very fast without any evidence of voltage or current artifacts (see Methods). We did not systematically employ this configuration because of the possibility that small dislocations might damage the cell. However, when we did obtain such recordings (see Fig. 7), it was clear that the rate of rise at the onset of the K signal was not decreased in the second response. This suggests that the kinetics of K efflux were not greatly changed during the initial part of the second pulse despite a significant increase in the time taken for the net

outward current to reach a maximum. Fig. 7 does reveal a decreased rate of rise in the latter half of the K signal from the second pulse. This is to be expected from our hypothesis that the long-term depression of the net outward current results from a specific depression of the slower Ca dependent component of ($I_{K(Ca)}$). Without $I_{K(Ca)}$ the net K current frequently declines during the 100 msec pulse as evidenced by current trajectories from Co-treated neurones (see Fig. 6, column 2).

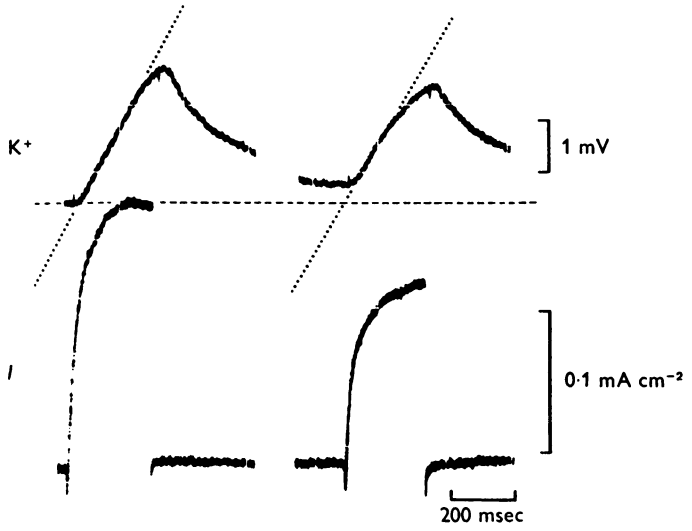

Fig. 7. The K signal (upper record, K^+) and the pipette current (lower record, I) during two voltage clamp depolarizations (separated by 800 msec) of 68 mV from −48 mV holding potential from a particularly favourable recording in which the K electrode was very near the membrane and the K signal shows little time lag. As indicated by the dotted lines, the initial rate of rise of the extracellular K was similar following the two pulses despite the depression and slowed onset in the second current trajectory resulting from repetitive activation. The rapid rate of rise indicates that the outward K current is unaltered during the initial part of the pulse and that the changes in kinetics noted on the current records are due to the increased effectiveness of the short-circuiting inward current.

Both the deficit in net charge transfer and the current deficit defined by trajectory analysis are abolished by Co treatment (Fig. 6, column 2). There is only a small decrease in net outward current in the second and third pulses (Fig. 6*A*, column 2), and the subtraction of pulse 1 from the normalized pulse 3 current, which gave the largest current deficit before Co treatment, does not reveal a significant inward component (Fig. 6*B*, column 2). The lack of an inward current is confirmed by the K activity data. There is little decrease in the K flux for pulses 2 and 3. Indeed, the calculated charge transfer deficit is −4% for pulse 2 and +5% for pulse 3 in this example. Additional trials in the same experiment and in other

experiments in the presence of Co also gave charge transfer deficits near zero.

The increase in an inward current component with repetitive activation in normal Ringer is also revealed by the direction of the tail currents when the membrane is returned to the holding potential after the pulse. The tail appears to consist of at least two components. One is very rapid with a time constant of about 2 msec and is not resolved well by the computer (but see Figs. 13 and 14). Following the first pulse the tail current is outward as shown in the computer averaged records in Fig. 8*A*, and with repetitive

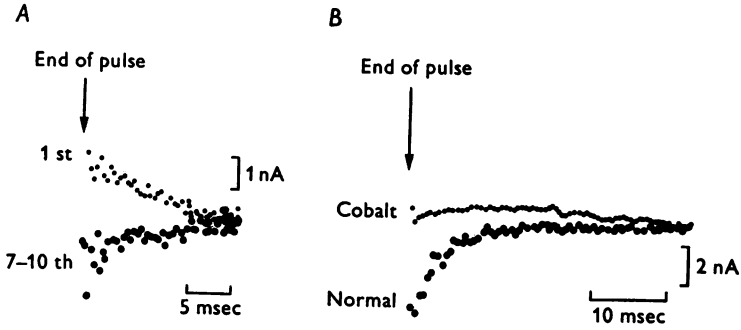

Fig. 8. Computer averaged tail currents following repolarizations to −50 mV from pulses to +7 mV. *A*, a comparison of the tail following the first pulse ('1st') with the average of tails following the last four pulses ('7–10th') in series of ten 100 msec depolarizations (here at 400 msec intervals) shows that the tail is outward-going at the beginning of the series but inversed to a net inward-going tail by repetitive stimulation of a typical neurone. Each record represents the average of sixteen sweeps. *B*, a comparison of the tail currents averaged for four 10 pulse series in another neurone before and after substitution of Co for Ca and Mg in the Ringer solution shows that the predominant inward-going component of the tail currents in the Ca containing medium is abolished in Co-Ringer.

activation, the tail current becomes inward-going, as revealed by the averaged tails from the last four pulses in trains of ten pulses also included in Fig. 8*A*. Reduction of the driving force for K by extracellular K accumulation does not explain this finding since the larger K accumulation expected during prolonged pulses or the larger outward currents in the first pulses of a series result in smaller shifts of tail currents in an inward direction. The apparently disproportionate increase in the inward-going component of the tail with repetitive stimulation results from the combined effects of a depressed outward K current and the increased inward Ca current. The depression of the true K current alone appears insufficient to account for this increase especially in the fast tail component.

The lack of a persistent inward current in Co-Ringer is also revealed in

the tail currents. Computer averaged records in Fig. 8*B* show that the tail becomes inward-going during repetitive activation in normal Ringer solution but is largely outward-going in Co-substituted Ringer.

Time course of the inward Ca current

The deficit in net charge transfer measured in the second of two pulses and the current deficit found by subtraction of current trajectories are of approximately equal magnitudes (Lux & Eckert, 1974), although there are some differences depending on the method of normalizing subsequent pulses (see Methods). Regardless of the method of calculation used, however, the trajectories of the current deficit have approximately the same time course and differ only in the magnitude of $I_{\text{in slow}}$ at the end of the 100 msec pulse. The time course of the current deficit trajectory resembles the time course of the net inward Ca current at lower voltages, i.e. with a slow onset and slow decay (Eckert & Lux, 1976). In order to test whether the current deficit curves of Fig. 6*B* represents the true time course of the Ca inward current, several stimulus paradigms were investigated.

Dependence of Ca current on pulse duration

The decay in magnitude of the current trajectory in Fig. 6*B* predicts that the Ca current should be less in a prolonged pulse (e.g. $\geqslant$ 500 msec) than in the sum of a series of shorter pulses (e.g. $\leqslant$ 100 msec). This prediction has been tested in a large number of fast and slow bursters. A typical result is shown in Fig. 9. The total duration of depolarizing voltage clamp (from -50 to $+7$ mV) is the same for the two trials, presented either as a continuous 1000 msec pulse or as ten 100 msec pulses, separated by 300 msec repolarizations. The net outward current is greatly depressed by the repetition of short pulses. Further, the rise time of the current trajectory becomes progressively slower. Although the total net outward current was decreased by 55 % for the repetitive compared with the continuous pulse stimulation, the K signal was only decreased by 46 % (see Fig. 9), resulting in a 17 % deficit in net charge transfer in the sum of short pulses compared with the total for the long pulse. Similar results were obtained in all experiments. The deficit in net charge transfer for short pulses compared with a long continuous one was 24 ± 8 % S.D. for the twenty-three experiments with short pulses of 100 msec spaced at 200–1000 msec intervals. With this range of interstimulus intervals, then, the repeated depolarizations do increase activation of the Ca current, producing a larger total inward flow of Ca^{2+} ions than occurs during a long pulse. However, the increased deficit with repeated depolarizations is often greater than expected from the summing of deficits equal to those of the pulse 2–pulse 1

calculation. This is due to the facilitation of the inward Ca current with repetitive activation.

If the Ca current is activated with repetitive depolarization, then the time course of the curve in Fig. 6*B* suggests that pulses shorter than 100 msec should contain a substantially greater deficit than found in long pulses. In another series of experiments the dependence of the total deficit

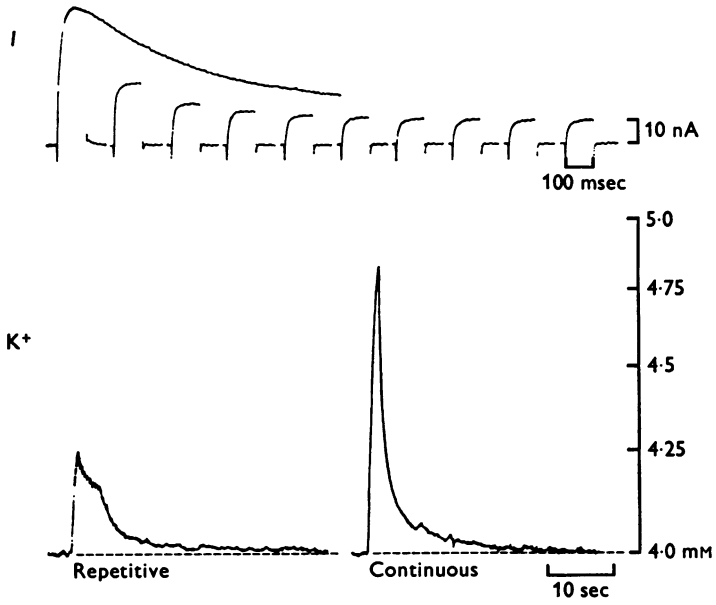

Fig. 9. Responses of a neurone to 1000 msec of voltage clamp depolarization (from −50 to +7 mV) presented either as a single pulse (continuous outward current response, upper trace) or as a set of ten 100 msec pulses, which are displayed in the discontinuous current record. In this example the repolarization interval between the pulses was 300 msec (not shown). The total time integral of the net outward current with repetitive stimulation is 45% of that with maintained depolarization (upper traces). The K efflux measured at the soma surface (lower traces) is decreased to 54% by repetitive activation, resulting in a calculated deficit of 17% of the long pulse current. These responses are typical of the fast burster studied.

current on stimulus duration was considered in more detail. Again a total of 1000 msec depolarization was given in trains of 25 msec (40 times), 50 msec (20 times), 100 msec (10 times) or 500 msec (2 times) pulses and the magnitude of the total deficit was compared with that of a continuous 1000 msec pulse. The results of these experiments summarized in Fig. 10*A* show a decreasing deficit with increasing stimulus durations from 25 or 50 msec.

Dependence of the Ca current on frequency of stimulation

The current trajectory for the deficit in Fig. 6*B* and the results of increasing pulse duration (Fig. 10*A*) imply at least partial inactivation of the Ca current with maintained depolarization and removal of this inactivation with a return to the holding potential (−50 mV). This

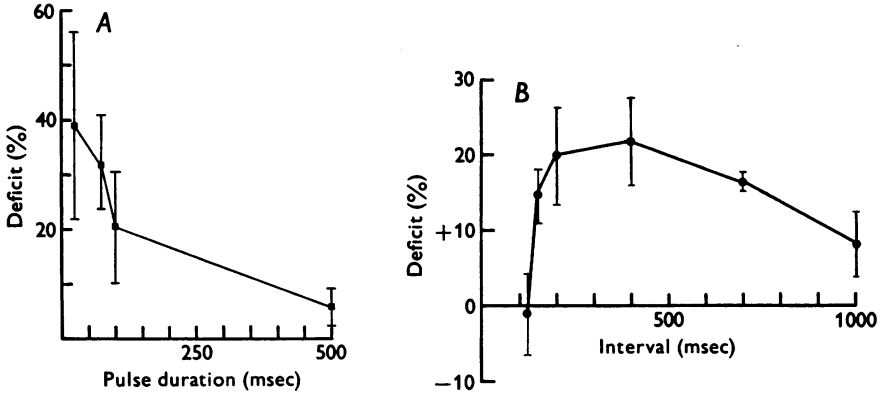

Fig. 10. *A*, dependence of the magnitude of net charge transfer deficit on the duration of the depolarizing pulse indicating partial inactivation with prolonged depolarization. A total of 1000 msec in voltage clamp depolarization were presented as forty 25 msec, twenty 50 msec, ten 100 msec or two 500 msec pulses (from −50 to +7 mV) with repolarization intervals of 300–500 msec. The resultant sum of net outward currents and K efflux were compared with those during a sustained 1000 msec pulse of the same magnitude. Vertical bars indicate standard deviations for three to six trials on each of the four neurones for which complete data were available. *B*, The magnitude of the deficit in net charge transfer in ten 100 msec pulses presented in trains of different interstimulus intervals as a function of intervals. The sum of net outward currents and K efflux during each train was compared with values from a sustained 1000 msec pulse of the same magnitude (from −50 to +7 mV). Vertical bars indicate the standard deviation of values from two to five trials in each of five fast bursters for which complete data were available.

suggests that the total Ca current with repetitive depolarizations should be frequency dependent. Trains of ten 100 msec pulses were tested at different interpulse intervals to determine the minimum interval for the removal of inactivation (to the level normally occurring at the −50 mV holding potential). The total deficit current as a function of repetition rate is presented in Fig. 10*B*. For the five experiments represented here, the maximum total Ca current occurred at repetition intervals between 200 and 400 msec. This implies that 100–300 msec at −50 mV are required to

remove the inactivation caused by transient (100 msec) depolarization (to levels between 0 and +20 mV).

The decrease in deficit with short interpulse intervals is reflected in the current deficit. In Fig. 11 the differences between pulse 1 and normalized pulse 2 currents are given for 120 and 400 msec intervals. The trajectory for the 400 msec interval is similar to that at a 1000 msec interval in Fig. 6*B*. In contrast the trajectory for the 120 msec interval does not reveal any substantial inward component. Indeed, there is a net outward

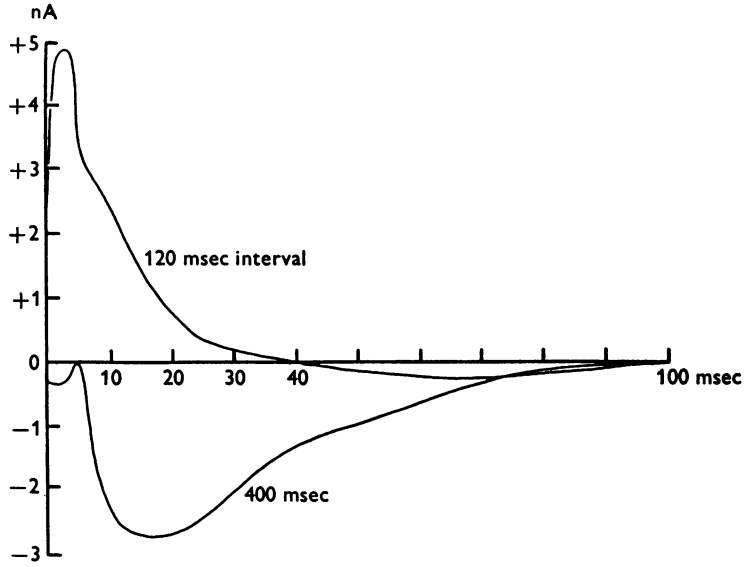

Fig. 11. Current deficits calculated by subtracting pulse 1 current trajectories from pulse 2 trajectories normalized to the height of pulse 1 at 100 msec (see Methods) for two sets of pulses. The calculated inward current seen when the onset of depolarization was separated by 400 msec intervals disappears at 120 msec intervals (see text for further discussion). Figures 12, 13 and 14 represent data from a single neurone typical of the fast bursters studied.

component. The latter may be partly due to the existence of a Ca inward current in pulse 1, which is still inactivated at the onset of pulse 2. However, for this short interval a deficit in net charge transfer is frequently measured and net inward tails developed with repetitive stimulation. This suggests that at least part of the trajectory may be due to a change in the kinetics of the K urrent in the second and subsequent pulses. Just such a change is discussed in the following paper (Heyer & Lux, 1976*b*).

Facilitation and defacilitation of the Ca current: frequency dependence

Failure to remove inactivation can account for the decrease in the total Ca current with < 100 msec between depolarizations. However, simple properties of inactivation do not explain the peak in Ca influx at 200–400 msec and the subsequent decrease in total Ca current with longer repetition intervals (⩾ 400 msec) as also seen in Fig. 10*B*. The graph suggests that there is a property of $I_{\text{in slow}}$ which opposes the effects of inactivation.

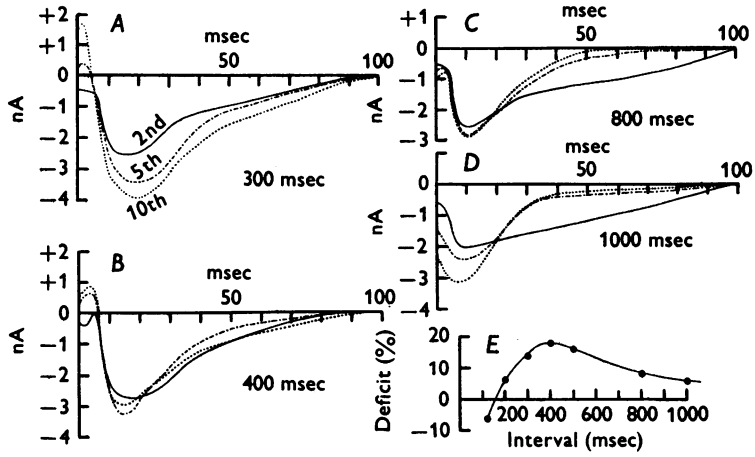

Fig. 12. Changes in the deficit during series of repetitive stimulations at different stimulus intervals for a single neurone. *A–D*, current deficits calculated by subtracting current trajectories of the first pulse from normalized trajectories of the second, fifth and tenth pulses (see Methods). Results are represented by different symbols: ——(second–first) ·—·—·(fifth–first) and ----- (tenth–first). Note facilitation of the inward current at 300 msec intervals and progressive defacilitation after the second pulse with repetitions at 800 and 1000 msec (intervals as noted). *E*, net charge transfer deficit as a function of the stimulus interval for this neurone. Currents and K efflux measured for the sum of ten 100 msec pulses are compared with the response to a single 1000 msec depolarization (from −50 to +7 mV).

Indeed, at intermediate intervals there is facilitation of the Ca current with repetitive depolarizations. Such an increase in $I_{\text{in slow}}$ is measured as an increase in net charge transfer deficit. Examples have been given (Figs. 2 and 6) showing progressive increases in calculated current deficits with repetitive stimulation.

The frequency dependence of these changes has been studied. Results from one neurone are shown in Fig. 12. For this particular neurone, the maximum Ca current for repetitive 100 msec pulses occurred at 400 msec

intervals (Fig. 12*E*). The increase in deficit at 400 msec is due to facilitation as well as to the removal of inactivation seen at shorter intervals. Facilitation with repeated depolarization is obvious at 300 msec intervals. In Fig. 12 the pulse 1 trajectory has been subtracted from normalized pulses 2, 5 and 10 of the 10 pulse series, demonstrating that the Ca current is increased with repetitive stimulation at this interval. No facilitation of pulses subsequent to pulse 2 is obvious at 400 msec intervals (Fig. 12*B*); the Ca current appears to be maximally facilitated at this interval.

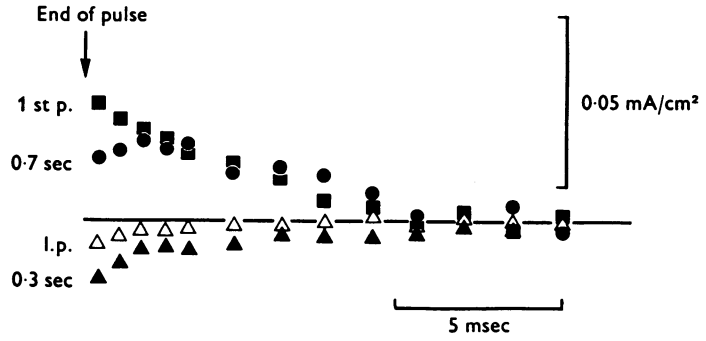

Fig. 13. Tail currents recorded after repolarization (to −50 from +7 mV) following a long pulse and short pulses presented at different frequencies. At the end of a 100 msec pulse (i.e. the first pulse in a series – '1st p.' = ■) the tail is outward. The average tail current from the last four pulses of a series of 700 msec intervals is also outward-going ('0·7 sec' = ●) reflecting some defacilitation of the current of this frequency (cf. Fig. 12*C*, *D*). With intermediate frequencies of stimulation the inward-going tail currents increase as shown in the averaged recordings of the last four pulses of a series of ten pulses at 300 msec intervals ('0·3 sec' = ▲). The tail is also inward after a 1000 msec long pulse ('l.p.' = △). Trajectories represent computer averages of tails from 12 pulses.

At even longer intervals a defacilitation with repetitive stimulation appears. With 800 or 1000 msec (Fig. 12*D*) the defacilitation appears first as a decrease in the magnitude of the pulse 2–pulse 1 trajectory (continuous lines) when compared with the corresponding curve at 400 msec intervals, for example, and then as a progressive decrease of the Ca current with repetitive stimulation beyond the second pulse.

Facilitation and defacilitation of the Ca current are also reflected in the tail currents. Computer averages of tail currents from the neurone of Figs. 11 and 12 are presented in Fig. 13. At the end of the first 100 msec pulse the tail is outward. With repetitive activation at a frequency with a large inward current (e.g. 300 msec) the tail becomes inward during subsequent pulses. Finally, with repetitive activation at even longer intervals (e.g. 700 msec) the tail becomes smaller but remains outward. A small but inward-

going tail also followed return to base line after 1000 msec continuous depolarization. With longer intervals defacilitation of the Ca current was inferred from the decreased charge transfer deficit and from the projected time course of the current deficit. Although the approach of the current deficit trajectory to zero at 800 msec is in part an artifact of the method for calculating the deficit (see Methods), the failure of the tail to become negative with repeated activation at 700–800 msec intervals suggests that there may be little or no Ca current by the ends of the last five pulses.

Effects of prepulses on the Ca current

In order to further investigate the activation of the Ca current with depolarizations to 0 mV or greater, the effects of prepulses (22 or 45 mV, 100 msec duration) immediately preceding the 100 msec depolarizing test pulses were assayed.

The effects of hyperpolarizing prepulses on the deficit varied considerably among the neurones studied. In all neurones the net outward current and the K^+ efflux were increased by the hyperpolarizing prepulses, regardless of the repetition rates of the pulse pairs. In most neurones the hyperpolarizing prepulses produced a decrease in the measured deficit. An example of this type of neurone is presented in Fig. 14. Current trajectories for the first, second and tenth 100 msec pulses, the current deficit (normalized pulse 10–pulse 1 trajectory) and the tail currents for depolarizing pulses from -50 to $+7$ mV (without prepulses) are given in Fig. 14*A*, *B* and *C*. With hyperpolarizing prepulses to -95 mV (100 msec duration) the net outward current is increased (Fig. 14*D*) and the calculated deficit in net charge transfer is decreased to 9 % (compared with 24 % in the absence of prepulses). Similarly, the current trajectory analysis shows less inward current in the first pulse preceded by a prepulse when compared with the control (Fig. 14*F*) and less facilitation of the inward current with repetitive stimulation (compare Fig. 14*B* with 14*E*). In addition, the tail currents which became inward with repetitive control stimulation remained outward in the presence of hyperpolarizing prepulses (compare Fig. 14*C* with 14*G*). Thus, the indicators from the current trajectories reflect the decrease in inward Ca current calculated from the net charge transfer and K efflux data. Decreases of up to 25% in the deficit current were produced by 22 or 45 mV hyperpolarizing prepulses in different neurones, but the effect was variable. In twenty-two series of tests on neurones with deficits ranging from 18 to 32%, hyperpolarizing prepulses decreased the deficit by 16 % ± 5 (S.D.).

The effects of depolarizing prepulses were more consistent: the deficit in net charge transfer, calculated from voltage clamp and K efflux data, was increased by the depolarizing prepulses. The increase was usually greater

with 45 than with 22 mV prepulses. With twenty-five series of tests on neurones with deficits ranging from 18 to 32%, depolarizing prepulses increased the deficit by 22% ± 5 (S.D.). This pattern is also revealed by the neurone in Fig. 14. The decrease in net outward current (see representative

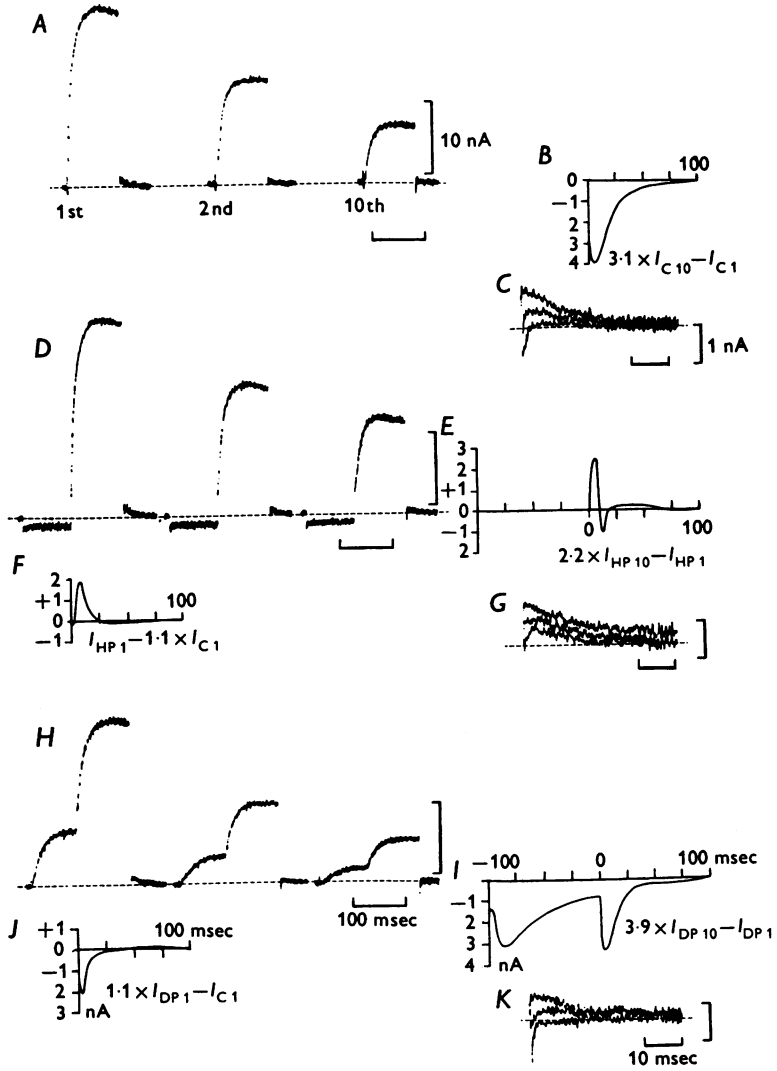

Fig. 14. For legend see facing page.

trajectories in Fig. 14*H*) was greater than the decrease in K efflux, resulting in net charge transfer deficit of 37% with depolarizing prepulses. However, the inward tail currents did not increase conspicuously (see Fig. 14*K*). The current trajectory analysis reveals that the deficit during the

first pulse was increased compared with the control (see Fig. 14*I*) and that the increase in inward current with repetition of the pulse pairs is nearly normal (compare Fig. 14*C* with 14*I*). There is also a significant deficit during the prepulse itself and this is strongly increased during repetitive activation (Fig. 14*I*). The larger percentage deficit with 45 mV prepulses than with 60–85 mV test pulses is predicted by the voltage dependence of the deficit discussed earlier (see Fig. 5) and accounts for much of the increase in total deficit. However, the results also indicate that the optimum repolarization level for $I_{\text{in slow}}$ magnitude and facilitation is less than or equal to -50 mV for most neurones and that these parameters can be decreased with increasing hyperpolarization in some neurones.

DISCUSSION

$I_{\text{in slow}}$ *in neurones of* Helix pomatia

The results of these experiments strongly suggest that the net inward current recorded in some *Helix pomatia* neurones during small depolarization (Eckert & Lux, 1975, 1976) and the deficit current measured in these neurones during larger depolarizations (when the net current is outward) results from the same mechanism. Ca^{2+} is the principle charge carrier of this slow inward current, $I_{\text{in slow}}$, at low voltages (Eckert & Lux, 1976) and Ca most probably carries the deficit current. As was demonstrated previously

---

Fig. 14. The effect of prepulses (100 msec duration, ± 45 mV from -50 mV holding potential) on responses to test voltage clamp depolarizations (100 msec; to $+7$ mV). *A*, *D* and *H*, current trajectories of the first, second and tenth pulses of the control (*A*) depolarizations preceded by hyperpolarizing (*D*) or depolarizing (*H*) prepulses. *B*, *E* and *I*, calculated current deficit trajectories (see Methods) representing changes in the inward current with repetitive stimulation. The pulse 1 trajectory was subtracted from the normalized pulse 10 trajectory for control depolarizations (*B*) preceded by hyperpolarizing (*E*) and depolarizing (*I*) prepulses (normalization and subtraction includes prepulses for the 100 msec preceding the test depolarization in *E* and *I*). Hyperpolarizing prepulses prevent an increase in inward current with repetitive stimulations (*E*) whereas a near normal increase in inward current in the tenth pulse preceded by depolarization is seen (*I*). Note also the large increase in inward current with repetition of the prepulse in *I*. *C*, *G* and *K*, tail currents following repolarization from the first (upper traces), second (middle traces) and tenth (lower traces) pulses of control (*C*) pulses preceded by hyperpolarization (*G*) or depolarization (*K*). *F* and *J*, current trajectories representing normalized subtraction of the first control pulse from the first test pulse preceded by hyperpolarization (*F*) or from the first test pulse preceded by depolarization (*J*). The hyperpolarizing prepulse slightly decreases the inward current (*F*) whereas the depolarizing prepulse increases the inward current (*J*).

for double pulses at fixed intervals (Lux, 1976), the deficit is abolished by external Co ions, which are either shown to block Ca currents (Hagiwara & Takahashi, 1967) or stimulus dependent increases in $[Ca]_i$ (Stinnakre & Tauc, 1973; Baker & Glitsch, 1975). With increasing depolarization it does not disappear except with voltages exceeding the equilibrium potential of all major ions (including Na, with an equilibrium potential of about +70 mV, calculated from the data of Thomas, 1972) except Ca.

It is not clear how selective the channels are for Ca. Our deficit measurements show a minimum at a membrane potential of +115 mV. If this is taken to be the equilibrium potential of the slow inward current, then it is less the value reported by Meech & Standen (1975) for Ca currents in these neurones. However, the calculations by the latter authors (i.e. based on the comparison of I–V plots before and after blocking the Ca current) would give an artificially high value if the methods used to block the Ca current also affected the Ca independent K current (Heyer & Lux, 1976*b*). Such non-specificity is shown for Co in the following paper (Fig. 4, Heyer & Lux, 1976*b*). Thus the relationship between the membrane potential at which we find the minimum deficit and the equilibrium potential for Ca ions is not yet clarified for these cells.

Ba and Mg (Eckert & Lux, 1976; H. D. Lux, unpublished observation) can substitute for Ca in $I_{\text{In slow}}$, indicating that the channel is certainly not entirely specific for Ca. In three experimental conditions we calculated a slight progressive increase in the deficit current with increasing depolarization: normal bursters depolarized above +150 mV (i.e. beyond the deficit minimum), and in the entire range of depolarizations in Co treated neurones and in two unidentified neurones which did not have an N-shaped I–V plot and responded like Co treated neurones. Whether or not these effects are due to the movement of ions through the $I_{\text{In slow}}$ channel or other effects, we cannot say. We have not investigated this interesting phenomenon.

In experiments reported here, the Ca current was always recorded in the presence of an outward K current. Even at very low levels of depolarization when we recorded a net inward current, there was a measurable K efflux. Thus, the original suggestion of Eckert & Lux (1976), that the inward Ca current can be analysed in isolation with small depolarizations appears to be incorrect. Indeed, a portion of the K current has thus far proved inseparable from the slow inward Ca current by usual procedures to remove one of the ionic fluxes (Heyer & Lux, 1976*b*).

The significance of this Ca current to neuronal behaviour for subthreshold depolarizations has been discussed (Eckert & Lux, 1976). It appears to play an important role in the generation of oscillatory waves in bursting pace-maker neurones and has properties appropriate for long-

term modulation of metabolism of membrane permeability. At the larger depolarizations considered in this report, $I_{\text{in slow}}$ shows several distinctive features, including frequency dependent inactivation, facilitation and defacilitation. We have observed that the slow burster (see Methods) has a relatively small inward Ca current when compared with the fast burster. Further differences in the time and voltage dependent characteristics of the delayed outward current in fast and slow bursters appear to reflect the relative magnitudes of the Ca current. In the following paper (Heyer & Lux, 1976*b*) we present data on the relationship of Ca ions in $I_{\text{in slow}}$ to the control of potassium permeability, and relate this to properties of slow and fast bursters.

The behaviour of the membrane conductance inferred from measurements of the slow inward Ca current described here differs significantly from membrane conductances to Na and K ions as previously described (Hodgkin & Huxley, 1952*a–d*). For example, there is an optimal holding potential for both the magnitude and facilitation of $I_{\text{in slow}}$. Hyperpolarization preceding a test pulse can reduce the magnitude of the inward current during the pulse, and preceding depolarization increases the inward current (see Fig. 14). Such behaviour contrasts sharply with the inactivation of currents in other neurones (e.g. Hodgkin & Huxley, 1952*c*).

Perhaps the most striking feature of $I_{\text{in slow}}$ is its facilitation. Our results reveal that facilitation can in principle be the property of a conductance or membrane permeability of Ca ions. The demonstration of a facilitating current corroborates and specifies the previous report of Lux & Eckert (1974). In a similar frequency range Stinnakre & Tauc (1973) found that the increase in intracellular Ca following each action potential increased during the burst in the parabolic pace-maker neurone of *Aplysia*. Here we have investigated in some detail stimulus parameters leading to the increase in inward current under voltage clamp conditions. For example, hyperpolarizing prepulses prevent the increase in $I_{\text{in slow}}$ with repetitive stimulation, whereas preceding depolarization does not prevent the increase (see Fig. 14). The time dependence of this increase in $I_{\text{in slow}}$ revealed characteristics familiar from facilitating and Ca depending systems, such as the synapse in which the processes supporting facilitation have not yet been defined (for references see Eccles, 1964; Zucker, 1974*a*, *b*). We report here that with controlled depolarizations an increase in the maximum Ca current depends on the frequency of stimulation. Evidence for partial inactivation at short interstimulus intervals (e.g. 20 msec), facilitation at intermediate intervals (e.g. 400 msec) and defacilitation at even longer intervals (e.g. 700 msec) has been presented.

We also report in the following paper that an increase in the internal concentration of Ca ions, as a result of $I_{\text{in slow}}$, leads to a long-term

depression of the K current through this channel. The frequency dependence of the depression of the K current shows a maximum depression for the same frequencies at which the Ca conductance is maximally facilitated. Thus, we might tentatively propose that facilitation is a consequence of the interactions between Ca and K ions in which the flow of K ions can impede the flow of Ca ions. Such a relationship between outward flow of K and inward flow of Ca would explain the unusual time and voltage dependence of the facilitation of $I_{\text{in slow}}$ reported in this paper. Hyperpolarization increases the K current and decreases the facilitation of $I_{\text{in slow}}$. Likewise facilitation of $I_{\text{in slow}}$ appears to be greater for small depolarizations in which the K flow is smaller (see Fig. 14*I* in which facilitation during 45 and 57 mV pulses are compared), and can be increased by depolarizing prepulses which decrease the flow of K in the test pulse.

Relationship of $I_{\text{in slow}}$ to other slow Ca currents

In squid giant axon two phases of Ca entry have been defined (see Baker & Glitsch, 1975, for references). One, the fast current, is blocked by tetrodotoxin and presumably uses the fast inward Na channel. The second is the 'slow current'. Slow tetrodotoxin insensitive inward Ca currents have been reported in a wide variety of membranes (Katz & Miledi, 1967, 1969; Trautwein, 1973; Keynes, Rojas, Taylor & Vergara, 1973; Mounier & Vassort, 1975*a*, *b*). Of these perhaps the slow Ca current of the squid giant axon and the Ca current proposed to mediate transmission in the squid giant synapse have been studied in the greatest detail. These currents are similar to $I_{\text{in slow}}$ in many respects. For example, they show comparable voltage dependences and are blocked by Co^{2+} and La^{3+} (Baker, Hodgkin & Ridgway, 1971; Katz & Miledi, 1967, 1969; Eckert & Lux, 1976; and experiments reported here). Both the late Ca current of the squid axon and $I_{\text{in slow}}$ at least partially inactivate with prolonged depolarization (Baker, Meves & Ridgway, 1973; Lux & Eckert, 1974; Eckert & Lux, 1975, 1976; and experiments reported here).

Synapses often show frequency dependent changes in efficacy (e.g. see Eccles, 1964, for references) which are similar to the frequency dependent changes in Ca currents reported here. Many of these aspects of synaptic plasticity have been attributed to variations in influx and internal concentrations of Ca and may, in fact, require the increase in Ca influx with repetitive stimulation (Zucker, 1974*b*).

REFERENCES

BAKER, P. F. & GLITSCH, H. G. (1975). Voltage dependent changes in the permeability of nerve membranes to calcium and other divalent cations. *Phil. Trans. R. Soc.* B **270**, 389–409.

BAKER, P. F., HODGKIN, A. L. & RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.* **218**, 709–755.

BAKER, P. F., MEVES, H. & RIDGWAY, E. B. (1973). Calcium entry in response to maintained depolarization of squid axons. *J. Physiol.* **231**, 527–548.

CARSLAW, H. S. & JAEGER, J. C. (1959). *Conduction of Heat in Solids*, pp. 510. Oxford: Clarendon.

ECCLES, J. C. (1964). *The Physiology of Synapses*. Berlin: Springer Verlag.

ECKERT, R. & LUX, H. D. (1975). A non-inactivating inward current recorded during small depolarizing voltage steps in snail pacemaker neurons. *Brain Res.* **83**, 486–489.

ECKERT, R. & LUX, H. D. (1976). A voltage-sensitive persistent calcium conductance in neuronal somata of *Helix*. *J. Physiol.* **254**, 129–151.

HAGIWARA, S. & TAKAHASHI, K. (1967). Surface density of calcium ions and calcium spikes in the barnacle muscle fiber membrane. *J. gen. Physiol.* **50**, 583–601.

HEYER, C. B. & LUX, H. D. (1976*a*). Facilitation and defacilitation of a calcium current in bursting pacemaker neurons in the snail *Helix pomatia*. *Pflügers Arch. ges. Physiol.* **362**, R40.

HEYER, C. B. & LUX, H. D. (1976*b*). Control of the delayed outward potassium currents in bursting pace-maker neurones of the snail, *Helix pomatia*. *J. Physiol.* **262**, 349–382.

HODGKIN, A. L. & HUXLEY, A. F. (1952*a*). Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**, 449–472.

HODGKIN, A. L. & HUXLEY, A. F. (1952*b*). The components of membrane conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 473–496.

HODGKIN, A. L. & HUXLEY, A. F. (1952*c*). The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 497–506.

HODGKIN, A. L. & HUXLEY, A. F. (1952*d*). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500–544.

KATZ, B. & MILEDI, R. (1967). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol.* **192**, 407–436.

KATZ, B. & MILEDI, R. (1969). Tetrodotoxin-resistant electric activity in presynaptic terminals. *J. Physiol.* **203**, 459–487.

KEYNES, R. D., ROJAS, E., TAYLOR, R. E. & VERGARA, J. (1973). Calcium and potassium systems of a giant barnacle muscle fibre under membrane potential control. *J. Physiol.* **229**, 409–455.

KERKUT, G. A. & MEECH, R. W. (1966). The internal chloride concentration of H and D cells in the snail brain. *Comp. Biochem. Physiol.* **19**, 819–832.

LUX, H. D. (1976). Simultaneous measurements of extracellular potassium-ion activity and membrane currents in snail neurons. In *Ion and Enzyme Electrodes in Biology and Medicine*, ed. KESSLER, R. Munich: Urban and Schwarzenberg Verlag.

LUX, H. D. & ECKERT, R. (1974). Inferred slow inward current in neurones. *Nature, Lond.* **250**, 574–576.

LUX, H. D. & HEYER, C. B. (1975). Fast K^+ activity determinations during outward currents of the neuronal membrane of *Helix pomatia*. *Bioelectrochem. Bioenerg.* **3**, 169–182.

LUX, H. D. & NEHER, E. (1973). The equilibration time course of $[K^+]_0$ in cat cortex. *Expl Brain Res.* **17**, 190–205.

MEECH, R. W. & STANDEN, N. B. (1975). Potassium activation in *Helix aspersa* under voltage clamp: a component mediated by calcium influx. *J. Physiol.* **249**, 211–239.

MOUNIÈR, Y. & VASSORT, G. (1975*a*). Initial and delayed membrane currents in crab muscle fibre under voltage-clamp conditions. *J. Physiol.* **251**, 589–608.

MOUNIÈR, Y. & VASSORT, G. (1975*b*). Evidence for a transient potassium membrane current dependent on calcium influx in crab muscle fibre. *J. Physiol.* **251**, 609–625.

NEHER, E. & LUX, H. D. (1969). Voltage clamp on *Helix pomatia* neuronal membrane: Current measurement over a limited area of soma surface. *Pflügers Arch. ges. Physiol.* **311**, 272–277.

NEHER, E. & LUX, H. D. (1973). Rapid changes of potassium concentration at the outer surface of exposed neurons during membrane current flow. *J. gen. Physiol.* **61**, 385–399.

REUTER, A. (1973). Divalent cations as charge carriers in excitable membranes. *Prog. Biophys. molec. Biol.* **26**, 1–43.

ROJAS, E. & ARMSTRONG, C. M. (1971). Sodium conductance activation without inactivation in pronase-perfused axons. *Nature, New Biol.* **229**, 177–178.

SAKHAROV, D. A. & SALANKI, J. (1969). Physiological and pharmacological identification of neurons in the central nervous system of *Helix pomatia* L. *Acta physiol. hung.* **35**, 19–30.

STINNAKRE, J. & TAUC, L. (1973). Calcium influx in active *Aplysia* neurones detected by injected aequorin. *Nature, New Biol.* **242**, 113–115.

THOMAS, R. C. (1972). Intracellular sodium activity and the sodium pump in snail neurones. *J. Physiol.* **220**, 55–71.

TRAUTWEIN, W. (1973). Membrane currents in cardiac muscle fibres. *Physiol. Rev.* **53**, 793–835.

ZUCKER, R. S. (1974*a*). Crayfish neuromuscular facilitation activated by constant presynaptic action potentials and depolarizing pulses. *J. Physiol.* **241**, 69–89.

ZUCKER, R. S. (1974*b*). Characteristics of neuromuscular facilitation and their calcium dependence. *J. Physiol.* **241**, 91–110.